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The adaptor 3BP2 is required for KIT receptor expression and human mast cell survival

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Abstract

3BP2 is a cytoplasmic adaptor protein that acts as a positive regulator in mast cell FcεRI-dependent signaling. The KIT receptor whose ligand is the stem cell factor (SCF) is necessary for mast cell development, proliferation and survival as well as for optimal IgE-dependent signal. Activating mutations in KIT have been associated with several diseases including mastocytosis. In the present work, we found that 3BP2 silencing impairs KIT signaling pathways, thus affecting PI3K and MAP kinase pathways in human mast cells from HMC-1, LAD2 (human mast cell lines) and CD34⁺-derived mast cells. Unexpectedly, silencing of 3BP2 reduces KIT expression in normal human mast cells as well as in HMC-1 cells where KIT is mutated, thus increasing cellular apoptosis and caspase 3/7 activity. 3BP2 silencing reduces KIT transcription expression levels. Interestingly, 3BP2 silencing decreased MITF expression, a transcription factor involved in KIT expression. Reconstitution of 3BP2 in knockdown cells leads to reversal of KIT expression as well as survival phenotype. Accordingly MITF reconstitution enhances KIT expression levels in 3BP2 silenced cells. Moreover, downregulation of KIT expression by miRNA221 overexpression or the proteasome inhibitor bortezomib also reduced 3BP2 and MITF expression. Furthermore, KIT tyrosine activity inhibition reduced 3BP2 and MITF expression, demonstrating again a tight and reciprocal relationship between these molecules. Taken together, our results show that 3BP2 regulates human mast cell survival and participates in KIT-mediated signal transduction by

directly controlling KIT receptor expression, suggesting its potential as a therapeutic target in mast cell-mediated inflammatory diseases and deregulated KIT disorders.

INTRODUCTION

Mast cells are key effectors in IgE-dependent hypersensitivity reactions, as well as in allergic and inflammatory disorders. Ligation of the high affinity receptor for IgE (FcεRI), constitutively expressed on mast cells, promotes cell activation and immediate release and production of pro-inflammatory mediators (1, 2). FcεRI-mediated mast cell activation can be dramatically enhanced by concurrent activation of KIT (CD117), a tyrosine kinase type III that plays a role in cell survival, proliferation and differentiation (3, 4). KIT binds its natural ligand, stem cell factor (SCF), resulting in receptor dimerization and activation of protein kinase activity. The activated receptor becomes autophosphorylated at tyrosine residues that serve as docking sites for signal transduction molecules containing SH2 domains. KIT activates AKT, Src family kinases, phosphatidylinositol 3-kinase, phospholipase C gamma, and Ras/mitogen-activated protein kinases (5). Subsequent activation of these signaling enzymes as well as the JAK-STAT pathway leads to mast cell growth, survival, chemotaxis and cytokine production (6). Dysregulation of KIT function (through gain of function mutations) results in certain pathologies like systemic mastocytosis, mast cell leukemias (7) and gastrointestinal stromal tumors (8).

SH3-binding protein 2 (3BP2) is a cytoplasmic adaptor originally identified as a protein that interacts with the SH3 domain of the protein tyrosine kinase (PTK) Abl (9). Human 3BP2 is a 561-aa protein containing an N-terminal pleckstrin homology (PH) domain, an SH3-binding proline-rich region, and a C-terminal SH2 domain. The 3BP2 encoding gene is located on human chromosome 4 (4p16.3 region). Mutations in the proline-rich region of 3BP2 are responsible for the autosomal dominant inherited disorder 'cherubism', which is characterized by excessive bone degradation of the upper and lower jaws, resulting in facial swelling (10). It has been reported that 3BP2 regulates bone homeostasis through osteoclast activation and osteoblast differentiation and function (11). 3BP2 is preferentially expressed in hematopoietic tissues where it contributes to the regulation of immune responses (12). 3BP2 regulates transcriptional activities via calcineurin- and Ras-dependent pathways in T lymphocytes (13). A positive regulatory role for 3BP2 in B cell receptor (BCR) function (14) has also been established, in that 3BP2-deficient mice show impaired optimal B cell activation and thymus independent humoral responses (15, 16). 3BP2 also plays an important role in NK cells, where it regulates cell-mediated cytotoxicity via its PH, SH2, and proline-rich regions (17). Moreover, phosphorylation of Tyr183 on 3BP2, which mediates the interaction with Vav-1 and PLC-γ, is critical for the ability of 3BP2 to positively regulate NK cell-mediated killing (17). We recently reported the essential role of 3BP2 in early and late events in FcεRI-dependent signaling in human mast cells (huMCs) (18). In the current work, we delve into the role of 3BP2 in KIT signaling and function in huMCs using an shRNA silencing approach. Our findings demonstrate that silencing of 3BP2 increases apoptosis and caspase 3/7 activity in human mast cells from CD34⁺ progenitors, LAD2 and HMC-1 cell lines. Moreover, 3BP2 regulates wild type and mutated KIT expression. 3BP2-GFP overexpression in 3BP2-silenced cells restores KIT expression

as well as cell survival. Interestingly, the expression of basic helix-loop-helix transcription factor microphthalmia associated transcription factor (MITF), an important regulator of KIT expression in mast cells, is decreased in 3BP2 knockdown cells. Furthermore the decrease in KIT expression following various treatments (use of proteasome inhibitor, bortezomib, and miRNA221 overexpression) leads to downregulation of 3BP2 and MITF expression. Finally, inhibition of KIT tyrosine kinase activity also reduces 3BP2 and MITF expression.

Altogether our data suggest a positive feedback loop between 3BP2 and KIT in mast cells.

MATERIAL AND METHODS

Cells and antibodies

The LAD2 human mast cell line, kindly provided by Drs. A Kirshenbaum and D.D. Metcalfe (NIH, Bethesda, MD, USA), was grown in StemPro-34 Serum-Free Medium (Invitrogen Life Technologies, Carlsbad, CA, USA), supplemented with StemPro-34 Nutrient plus L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 U/ml), and 100 ng/ml recombinant stem cell factor (SCF) (Amgen, CA, USA)(19). Primary huMCs derived from CD34-positive peripheral blood cells were obtained from healthy donors following informed consent using a protocol (98-I-0027; PI: Dr. A. Kirshenbaum) approved by the NIAID IRB, and differentiated *in vitro* for 8 weeks in the presence of 100 ng/ml IL-6 and 100 ng/ml SCF as previously described (20). After 8 weeks, culture purity was assessed by surface FcεRI and KIT expression. The human mast cell line HMC-1 was obtained from J.H. Butterfield (Mayo Clinic, Rochester, MN, USA) and was grown in Iscove's medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml)(21). Mouse antibodies, anti-3BP2 C5, anti-phospho AKT 1/2/3 (Ser 473)-R and anti-KIT (clone Ab81; unconjugated or PE conjugated) were purchased from Santa Cruz (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA). Anti-mouse Alexa-647 conjugated antibody was from Life Technologies. Anti-phospho STAT1 monoclonal (clone ST1P-11A5) antibody was from Zymed Laboratories (Invitrogen). Anti-pKIT Tyr 703 (D12E12), anti-p-ERK Thr202/Tyr204, anti-AKT and anti-MITF were purchased from Cell Signaling (Cell Signaling Technology, Inc. Danvers, MA, USA). Anti-α-tubulin (clone DM1A) and the tyrosine kinase inhibitor sunitinib malate were purchased from Sigma (Sigma, St. Louis, MO, USA). Anti-GFP antibody was from Roche (Roche Molecular Biochemical, IN, USA).

Lentiviral infection

Scrambled siRNA-GFP and SH3BP2-set piLenti-siRNA-GFP (sequences 1, 2, 3 and 4 in 184, 996 1067 and 1390 positions of the cDNA respectively) were purchased from Applied Biological Materials (Richmond, BC, Canada).

Lentiviral particles to silence 3BP2 gene expression were generated using Mission® shRNA technology according to the manufacturer's instructions (Sigma, St. Louis, MO, USA), and huMCs were infected as described elsewhere (18).

CD34⁺ peripheral blood-derived mast cells, LAD2 and HMC-1 cell lines were infected according to the manufacturer's instructions and selected with puromycin for 1 week. The

Lenti ORF clone of human SH3-domain binding protein 2 (SH3BP2), transcript variant 1, mGFP tagged, lenti ORF clone of human microphthalmia-associated transcription factor (MITF), transcript variant 1, mGFP tagged and Lenti vector with C-terminal monomeric GFP tag as a control (OriGene Technologies, Rockville, MD) were used for 3BP2 and MITF reconstitution experiments respectively.

Lentiviral particles expressing miRNA221, LentimiRa-GFP-hsa-mir-221 Virus, and Lenti-III-mir-GFP Control Virus were from Applied Biological Materials, Inc. (Richmond BC, Canada). LAD2 cells were infected according to the manufacturer's instructions and were selected with puromycin for 1 week.

Lentiviral particles carrying human KIT short hairpin RNA (shRNA) or no target (NT) control shRNA sequences were purchased from Santa Cruz Biotechnology. Infection of LAD2 cells and HMC-1 cells were done according to manufacturer's instructions and were selected with puromycin for 1 week.

KIT expression was analyzed by Western blot, and the silencing efficacy was determined by normalizing to α -tubulin.

RNA extraction and retrotranscription

Total RNA was extracted with an RNAeasy Mini Kit Qiagen (Qiagen, Hilden, Germany) from 2×10^6 non-transduced, non-target control and 3BP2 knockdown mast cells. cDNA was generated using an mRNA High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

Real-time PCR

Real-time PCR for KIT, GATA2, GATA3, SCL and MITF was performed using a TaqMan Gene Expression Assay (Applied Biosystems) on an ABI-Prism 7300 Sequence Detector (Applied Biosystems). 18S RNA amplification control was used for cycle normalization. Data were analyzed using 7500 SDS Software (Applied Biosystems). Analysis was performed using the 2^{-CT} method (22). All PCR reactions were set up in triplicate.

Cell activation, immunoprecipitation and immunoblotting

Cells were starved overnight in culture media without SCF. The following day, cells were stimulated with 100 ng/ml of SCF in Tyrode's buffer for the indicated times. Whole cell lysate preparations were obtained as described elsewhere (23). Immunoprecipitation experiments were conducted using a procedure described elsewhere (24). Using lentiviral technology signaling data was conducted after infection and 1 week after starting selection with puromycin (day 0).

FACS staining

KIT expression was detected by direct or indirect staining with the indicated Abs for 30 min at 4°C. In the case of the use of unconjugated KIT, cells were incubated afterwards with fluorochrome-conjugated secondary antibody for 30 min at 4°C. Cells were then analyzed using a FACSCalibur flow cytometer (FACScan; BD Biosciences, Mountain View, CA). In

all cases, dead cells were gated out based on the forward scattering (FSC) and side scattering (SSC) profile.

Detection of apoptosis and Caspase-3 and -7 activities

Apoptosis was measured using the APC Annexin V (BD Pharmingen, San Jose, CA, USA) according to the manufacturer's suggested protocol and analyzed by flow cytometry.

Caspase activity was assayed using the Caspases-Glo® 3/7 Assay (Promega, San Luis Obispo, CA, USA) according to the manufacturer's instructions.

Proteasome and tyrosine kinase inhibitor cell treatment

Cells were incubated in culture media with the proteasome inhibitor MG132 at 10 µM or vehicle (DMSO) for 3 hours at 37°C and KIT staining was analyzed by flow cytometry. Cells were treated with bortezomib at 1 and 10 µM or vehicle (DMSO). After 24 hours incubation with bortezomib cells were lysed as described elsewhere (18, 23). Cells were incubated with sunitinib malate at 1 and 10 µM or vehicle for various times (16 and 24 h). Cells were lysed as described previously and western blot techniques were carried out using the whole cell lysates to analyze KIT, 3BP2 and MITF expression.

Yeast three-hybrid assay

The yeast three-hybrid system was performed, as described elsewhere (25). The cytoplasmic tail of human KIT, cloned in the bicistronic pBridge vector (which carries the src-Fyn clone) was obtained after PCR using KIT full length d'Origene (NM-000222.1) as a template and the primers 5' C ACC GAA TTC ATG AAA TAT TTA CAG 3' and 5'-AAA GGA TCC TCA GAC ATC GTC G 3'. The PCR product was cloned in pBridge using EcoRI and BamHI. KIT-pBridge was cotransformed with 3BP2 cloned in pACT2 (26), in the yeast strain CG1945. CD244 cloned in pBridge (26) and Grb2 cloned in pGAD (27) were used as a positive control for 3BP2 and KIT respectively, and empty pGAD was used as a negative control. Yeast clones were then tested using the β-galactosidase assay. The β-galactosidase liquid culture assay using *o*-nitrophenyl β-D-galactopyranoside as a substrate was carried out as described in the Clontech yeast protocols handbook.

Data analysis

All results are expressed as mean ± standard deviation of the mean (SD). Student's *t*-test was used to determine significant differences (*p* value) between two experimental groups after determination of normal distribution of the sample and variance analysis.

RESULTS

3BP2 silencing impairs mutated KIT signaling in HMC-1

The adaptor 3BP2 was recently shown to be required for optimal IgE-dependent immediate and late mast cell responses such as degranulation and IL-8 or GM-CSF secretion (18). Mast cells are defined by their cell surface expression of the KIT receptor (3, 4), a receptor that can synergize with FcεRI signaling, thus increasing degranulation and cytokine release (28).

Here we explored the role of 3BP2 in KIT receptor signaling following lentiviral shRNA silencing of 3BP2 expression in HMC-1.

HMC-1 cells were transduced with various 3BP2 pi-Lenti-siRNA-GFP sequences and 3BP2 was analyzed by western blot (Figure 1A). We took advantage of the human mast cell line HMC-1, which expresses a constitutively active form of KIT. Two activating mutations in the proto-oncogene of c-kit cause its autophosphorylation and kinase activation, thereby inducing ligand-independent proliferation (29-31). KIT autophosphorylation in HMC-1 cells results in the permanent activation of other signaling pathways, including the Ras-MAPK-ERK and the PI3K pathways, which are involved in cell survival and proliferation (31). Although the basal phosphorylation of KIT and multiple downstream effectors was increased in these cells, due to SCF-independent activation, the addition of SCF at 5 minutes still caused some enhancement of the global phosphorylation. It has been reported that even in cells expressing c-kit^{D816V} (HMC-1 cell line harbors this mutation) there is still a need for SCF to promote activation of AKT and ERK1/2 (32). Interestingly, silencing of 3BP2 in HMC-1 cells resulted in a marked decrease in basal and induced phosphorylation of KIT and other effectors such as AKT, ERK and STAT 1 phosphorylation. Unexpectedly, but interestingly, the expression level of mutated KIT receptor decreased after 3BP2 silencing (Figure 1B). The surface expression of KIT, analyzed by flow cytometry, also showed a concomitant decrease (Figure 1C).

3BP2 silencing leads to downregulation of KIT signaling in LAD2 and CD34+ derived human mast cells

We confirmed the data obtained in HMC-1 cells using LAD2 cells as well as CD34⁺ peripheral blood-derived huMCs (CD34⁺ huMCs) in which KIT is not mutated. As shown in Figure 2, KIT phosphorylation was markedly reduced and its ability to activate PI3 kinase was also impaired as measured by phosphorylation of Akt, a surrogate marker of this pathway. In addition, ERK phosphorylation at 5 and 15 min following SCF addition was also impaired. 3BP2 silencing reduces KIT expression to a large extent in LAD2 cells and CD34⁺ huMCS compared to HMC-1. Given that KIT expression was downregulated in 3BP2-silenced cells, we investigated the possible interaction between these two molecules. The KIT cytoplasmic domain has three tyrosine residues (Y553, Y730, Y823) that follow the consensus Y (E, M, V) (N, V, I) X, where x denotes any amino acid. These tyrosine residues may constitute docking sites for the 3BP2 SH2 domain following their phosphorylation. Accordingly, SCF stimulation (for 2 or 15 min) of HMC-1 or LAD2 cells showed that 3BP2 co-immunoprecipitated with KIT. However, a more in-depth analysis using the three-hybrid approach between these two molecules showed no direct association (Supplemental Figure 1).

Silencing of 3BP2 results in increased apoptosis and caspase 3/7 activity

Our findings showed that 3BP2 silencing resulted in decreased phosphorylation of the serine/threonine kinase AKT. This kinase is activated downstream of PI3K and is involved in delivering survival signals in response to SCF (33). Thus, we explored the impact of 3BP2 silencing on mast cell survival. Annexin V staining was performed in CD34⁺ huMCs and LAD2 cells. As described elsewhere (18), CD34⁺ huMCs were infected in week 6 and

the efficacy of silencing as well as cell viability was checked two weeks later (experiment day 0). In the case of LAD2, after infection with 3BP2 shRNA for one week and selection in the presence of puromycin for an additional week, cells were assayed for cell survival. As shown in Figure 3, cells in which 3BP2 was silenced showed significantly increased Annexin V staining after 6 days in culture (Figure 3A, B, C). Further apoptosis was evaluated after day 6 by assaying functional caspase 3/7 activity after NT (non target) or 3BP2 shRNA transduction in LAD2 cells and HMC-1 cells. As expected 3BP2 silencing significantly induced caspase 3/7 activity compared to controls in all cell types (Figure 3D and 3E). Thus, the loss of 3BP2 expression has a marked effect on huMC survival. It is important to note that apoptosis was increased after day 6, and that all the signaling data shown in Figures 1 and 2 was collected on day 0 when the levels of cell death were comparable in control and 3BP2 silenced cells and no significant differences in cell viability were observed (Figure 3).

Silencing of 3BP2 results in downregulation of KIT mRNA expression

Next, we explored the ability of 3BP2 to regulate KIT expression. One possibility was that 3BP2 stabilizes KIT expression by preventing receptor proteasome-mediated degradation. To test this hypothesis we used the proteasome inhibitor MG132. If the hypothesis was correct, MG132 treatment would increase KIT cellular levels. However, following proteasome inhibition, KIT expression decreased in control cells compared to untreated cells, whereas in 3BP2-silenced cells KIT levels were undetectable (Figure 4A). Thus, the data indicate that prevention of proteasome degradation did not reverse the reduction in KIT expression in 3BP2-silenced cells, and conversely that receptor expression was significantly reduced in all treated cells. Interestingly, FcεRI expression showed no changes in any case at this time (data not shown). This raised the possibility that 3BP2 controls KIT expression at the mRNA level. Samples from control and 3BP2-silenced cells were analyzed for c-kit mRNA by real-time PCR. As shown in Figure 4 B, c-kit mRNA levels were reduced in 3BP2-silenced cells compared to control cells. Thus, the data indicate that 3BP2 is required for normal expression of c-kit mRNA.

Analysis of the transcriptional regulation of KIT expression in 3BP2-silenced mast cells

Since decreased c-kit mRNA levels could result from transcriptional or post-transcriptional regulation, we explored the effect of 3BP2 silencing in mast cells on various transcription factors involved in the regulation of KIT expression. The promoter region of human c-kit has been thoroughly investigated (34). It has been reported that gene expression of c-kit in mast cells requires GATA2 and Sp1 recruitment to the promoter region (35). Although a Th2 master regulator (36), GATA 3 has also been proposed to stabilize KIT expression in mast cells and promote mast cell development in the absence of Notch signals when IL-3 and SCF are present (37). In addition, the transcription factor SCL has been reported as necessary for KIT expression and function in hematopoietic cells (38). Another transcription factor, the basic helix-loop-helix microphthalmia associated transcription factor (MITF), binds to a CACCTG motif in the c-Kit promoter and has been shown to be an important regulator of KIT expression in mast cells (39) as well as melanocytes (40).

Analysis of the expression of GATA 2, GATA3, SCL and MITF by real-time PCR in control versus 3BP2-silenced cells revealed that the mRNA levels of GATA 2, GATA3, SCL and MITF were not decreased in 3BP2-silenced cells. Conversely, we found that the mRNA for most of these transcription factors increased in 3BP2-silenced mast cells, suggesting a compensatory mechanism activated by 3BP2-mediated KIT downregulation. The maintenance of expression of GATA 2, GATA 3, SCL and MITF mRNA after 3BP2 knockdown serves as well as an additional control to support 3BP2 shRNA specificity (Supplemental Figure 2).

The reciprocal regulatory relationship of KIT, 3BP2 and MITF as a possible link

Interestingly, recent findings suggest that there is also some inverse regulation, in that KIT signaling regulates MITF expression at the post-transcriptional level (41). Thus, reduction of KIT levels should reduce MITF expression. KIT expression is also regulated by miRNA 221 and 222 in murine mast cells differentiated from bone marrow (42, 43). To test this possibility in the context of 3BP2, KIT expression was silenced using miRNA 221 lentiviruses, and KIT, 3BP2 and MITF expression were evaluated by western blot in LAD2 cells. The results revealed a concomitant reduction in expression of KIT, 3BP2 and MITF under miRNA221 transduction (Figure 5A). Levels of miRNA 221 and miRNA GFP (as a control) transduction monitoring GFP expression as well as KIT expression in those cells were analyzed by flow cytometry (Figure 5B). We also manipulated KIT expression using bortezomib. The proteasome inhibitor bortezomib has been reported to cause oncogenic KIT downregulation by inhibiting the transcriptional machinery and blocking the degradation of pro-apoptotic factors, inducing pro-apoptotic activity in gastrointestinal stromal tumor (GIST) (44). After bortezomib treatment we found that KIT expression was severely impaired in mast cells. Moreover 3BP2 and MITF expression were also downregulated, again indicating the close regulation of these molecules (Figure 5C). To assess whether KIT tyrosine kinase activity was needed to regulate 3BP2 and MITF protein expression, cells were inhibited with sunitinib, a second-generation KIT inhibitor that is active in imatinib-resistant disease in GIST (45). Previously, MITF expression was reported to be reduced after imatinib inhibition (41). HMC-1 cells, in which KIT is mutated and constitutively active, were chosen for the analysis. Our data shows that inhibition of KIT tyrosine kinase activity also leads to a severe decline in 3BP2 and MITF expression (Figure 5D). KIT silencing approach also renders a decrease of 3BP2 expression (Supplemental Figure 3). Collectively, these results argue for a tight link in the regulation of KIT and 3BP2 involving MITF, and suggest that a common regulatory pathway may be in play in human mast cells.

3BP2 silencing leads to decreased MITF expression

We explored whether 3BP2, as a component of KIT signaling, might also play a role in regulating post-transcriptional levels of MITF. Analysis of the levels of MITF protein after 3BP2 silencing demonstrated decreased MITF levels compared to control-transduced HMC-1 cells (Figure 6). Various 3BP2 sequences show a decrease in 3BP2 expression concomitant with KIT and MITF expression. Our results suggest that MITF protein expression may be regulated by 3BP2, consistent with the marked reduction in KIT expression by 3BP2 silencing in these cells. However, we cannot rule out the possibility that other factors may be involved in 3BP2-dependent KIT expression.

3BP2 reconstitution restores KIT expression and cell survival

In order to analyze the direct and specific effect of 3BP2 on KIT expression and cell survival we reconstituted the cells with 3BP2-GFP. Cells were transduced with human SH3-domain binding protein 2 (3BP2), GFP tagged and Lenti vector with C-terminal GFP tag as the control. The percentage of infected cells was analyzed using GFP analysis and flow cytometry. KIT staining was performed from day 2 to 6. KIT expression was completely recovered (99%) in 3BP2-silenced cells after 3BP2 reconstitution at day 6 (Figure 7A and B). Apoptosis was assayed by analyzing caspase 3/7 activity on various days. The cell survival of reconstituted cells was similar to control cells at day 6 (Figure 7C), concomitant with the highest KIT expression levels. In order to evaluate MITF participation in KIT expression upregulation in the 3BP2 knock down cellular context NT (non-target) and 3BP2-silenced cells were reconstituted with MITF GFP tag. Our results showed an increase in KIT expression at day 6 in 3BP2 silenced cells suggesting an involvement of MITF in the 3BP2 dependent KIT expression regulation (Figure 7D). Thus, altogether our data show that 3BP2 is able to regulate KIT expression, at least through MITF, as well as human mast cell survival.

DISCUSSION

Here we report that 3BP2 is an important adaptor molecule for mast cell survival that acts by promoting KIT receptor signaling. This receptor-upon-ligand recognition initiates signals that are critical for mast cell growth, development, and survival (46). We found that silencing of 3BP2 in mast cell decreases PI3K and ERK1/2 signaling pathways and induces apoptosis as measured by Annexin V staining and increased caspase 3/7 activity. Unexpectedly, we also found a marked requirement for 3BP2 in the expression of KIT receptor. In addition, 3BP2 reconstitution restores KIT expression and cell survival. In order to understand how 3BP2 regulates KIT expression we explored various scenarios. One possibility is that 3BP2 stabilizes receptor expression by competing for binding with molecules that regulate KIT expression, such as c-Cbl. This molecule binds to various tyrosines (Y568, Y570 and Y936) in the KIT cytoplasmic domain, and regulates proteasomal degradation of this receptor (47). Although 3BP2 co-immunoprecipitates with KIT after SCF stimulation, our results point to an indirect association that may be mediated by common partners of both molecules, such as Grb2, PLC gamma, PI3K or an unidentified ligand (13, 17, 18, 48). This, however, does not preclude the possibility that in the absence of 3BP2, binding of c-Cbl to KIT could increase and hence, promote its degradation. However, treatment with the proteasome inhibitor MG132 did not revert KIT expression; conversely, the receptor expression levels were even more reduced. These findings are consistent with data from gastrointestinal stromal tumor (GIST) cell lines, in which KIT mutation confers an oncogenic phenotype, and treatment with bortezomib, an inhibitor of the ubiquitin proteasome machinery, leads to a marked reduction in KIT expression and the death of these cells (44). Interestingly, our data show that bortezomib treatment also reduces KIT as well as 3BP2 expression in mast cells, indicating tightly linked regulation of 3BP2 and KIT molecules. Moreover, a reduction in KIT transcription has been reported as an underlying mechanism for bortezomib-mediated inhibition of KIT expression (44). This is consistent with our findings that bortezomib inhibits 3BP2 expression and that decreased

levels of 3BP2 would lead to downregulation of c-kit mRNA. Interestingly, we found that the basic helix-loop-helix transcription factor microphthalmia associated transcription factor, MIFT, an important regulator of KIT expression in mast cells (39), is reduced after bortezomib treatment. Recent data show that there is also some inverse regulation, in that KIT signaling regulates MIFT protein expression levels. MIFT mRNA levels do not change significantly with KIT signaling, suggesting posttranscriptional regulation. The authors found that miR-539 and miR-381 are down-regulated by KIT signaling and they repressed MIFT expression through conserved miRNA binding sites in the MIFT 3'-untranslated region. (41). KIT is also a well-established target for a family of miRNAs comprising miR-221 and miR-222 (42). Our data show that miR-221-GFP overexpression in transduced LAD2 cells reduces KIT and MIFT expression; moreover 3BP2 expression is also reduced. This again suggests a common link among these molecules. It is important to take into consideration that miRNA-221 has been reported to downregulate several gene targets in mast cells involved in regulating degranulation, cytokine production, cell cycle and adherence (43, 45). Thus, the reductions in KIT, MIFT and 3BP2 following miR-221 overexpression may occur independently and the data should be considered with caution. Mutations in the 3'UTR of the KIT sequence that lead to miR-221 binding impairment and consequently post-transcriptional regulation have been found in systemic mastocytosis patients (43). Although there is no clear evidence that impairment of miR-221 binding is relevant in the disease, given the importance of this miRNA family in the regulation of mast cell biology and KIT expression it is highly likely that these mutations play a role in mast cell derived diseases. In humans the D816V mutation of KIT is highly prevalent and is associated with mast cell leukemia and mastocytosis (30, 49). Our finding that the adaptor 3BP2 may regulate KIT expression is of particular interest with regards to control of KIT D816V expression in mastocytosis. Specifically, substitutions at position D816 in the activation loop rendered the KIT kinase almost completely resistant to Imatinib (50), the kinase inhibitor used in the treatment of KIT oncogenic diseases (51). Thus, the apoptosis promoted by 3BP2 silencing in HMC-1 cells, where oncogenic KIT harbors this mutation, makes 3BP2 an attractive new potential target for developing new therapies. Inhibition of tyrosine kinase activity by sunitinib leads to a reduction in 3BP2 and MIFT expression while the levels of KIT expression remain detectable. Interestingly we found a decrease in MIFT protein expression after 3BP2 silencing and a KIT oncogenic reduction in HMC-1. Our findings suggest that 3BP2 is an important post-transcriptional regulator of the KIT-MITF axis, as we found that 3BP2 silencing led to a reduction in MIFT protein but not MIFT transcripts. This is a previously unrecognized link between MIFT and 3BP2 expression and further studies are needed to dissect the mechanism. MIFT is essential for osteoclast development and function (52) and 3BP2 is essential for coordinating bone homeostatic signals in both osteoclast and osteoblast lineages (53). In the bone metabolism context 3BP2 has been shown to regulate the bone marrow monocyte response to macrophage and osteoclast differentiation signals downstream of the receptors for macrophage colony-stimulating factor (M-CSF) and the receptor activator of nuclear factor kappaB ligand (RANKL) through mechanisms that involve control of extracellular signal-regulated kinase (ERK) and spleen tyrosine kinase (SYK) activity, respectively (11). In RAW264.7 cells it has been found that 3BP2 is a key regulator of RANK-mediated macrophage differentiation into osteoclasts through Src and NFATc1 activation (54).

Interestingly, MITF has been shown to reside downstream of the macrophage colony-stimulating factor (M-CSF) and receptor activator of NF- κ B ligand (RANKL) signaling pathways, which are critical for osteoclast proliferation, differentiation and function (55, 56). This suggests that further exploration of the link between MITF and 3BP2 in osteoclasts is warranted. Amino acid missense mutations in 3BP2 have been identified in several cherubism patients (57). Cherubism is a fibrous dysplasia-like syndrome characterized by excessive bone resorption in the jaw with accumulation of inflammatory/fibrous tissue. It has recently been reported that Tankyrase, a member of the poly (ADP-ribose) polymerase (PARP) family, regulates 3BP2 stability through ADP-ribosylation and subsequent ubiquitylation by the E3-ubiquitin ligase RNF146 in osteoclasts. Cherubism mutations uncouple 3BP2 from Tankyrase-mediated protein destruction, which results in its stabilization and subsequent hyperactivation of the Src, Syk, and Vav signaling pathways, explaining the pathological features observed in the disease (58, 59).

Collectively, our findings reveal a new role for 3BP2 as an important regulator of KIT receptor expression and function in mast cells. Previously we found that 3BP2 was required for early and late events in Fc ϵ RI signaling (18), so we propose a model in which 3BP2 would act as a link in the signaling transduction of both receptors. Uncovering the details of how 3BP2 regulates KIT expression and signaling will be essential in attempts to understand the therapeutic potential of 3BP2 as a target for mast cell proliferative diseases and those resulting from mutations of the KIT receptor.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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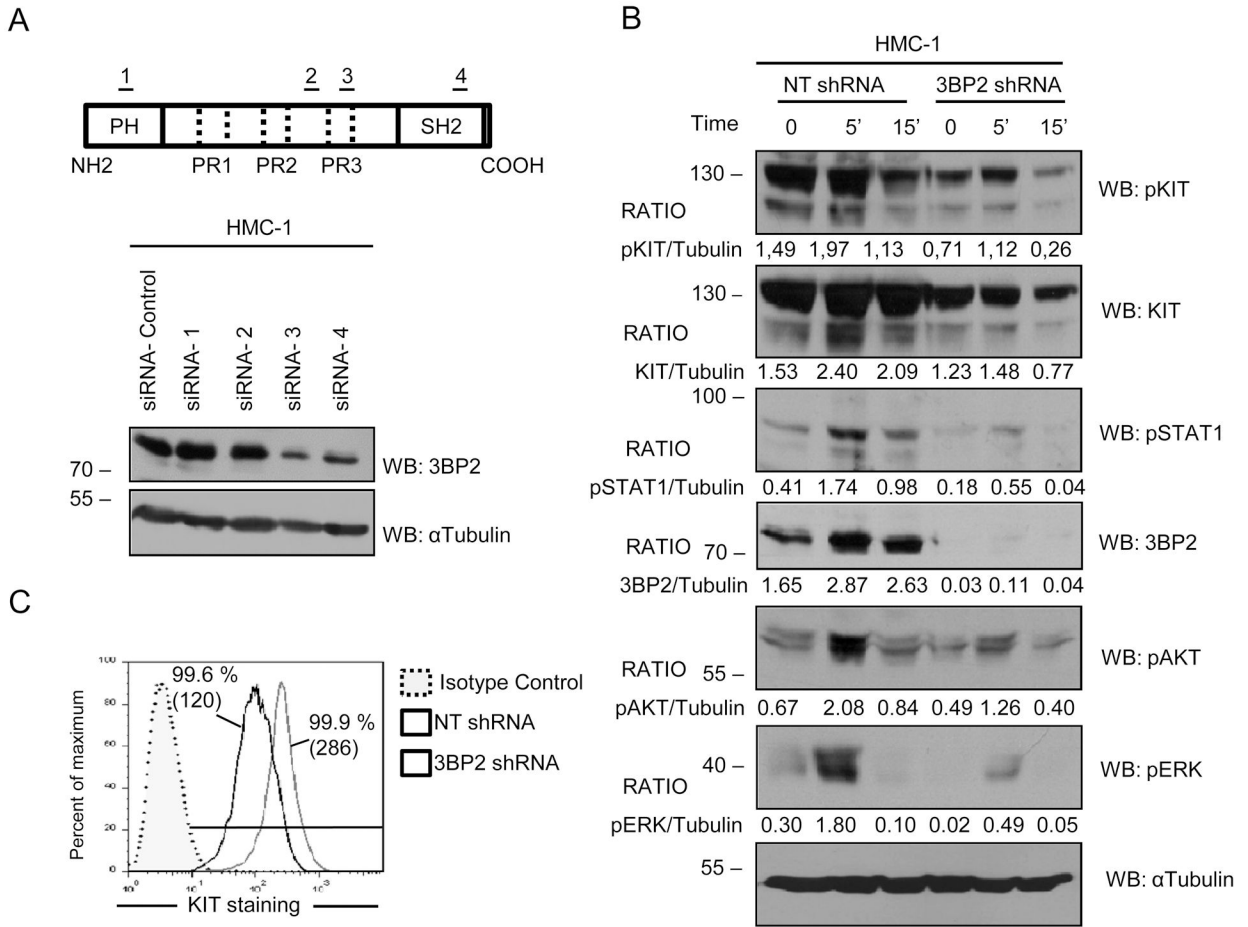


Figure 1. 3BP2 silencing impairs mutated KIT expression and signaling in HMC-1 cells
HMC-1 cells were transduced with various 3BP2 pi-Lenti-siRNA-GFP sequences as indicated in the 3BP2 scheme of the protein (first sequence in the PH domain of 3BP2 and the fourth sequence in the SH2 domain of the molecule) and in material and methods section and 3BP2 expression was analyzed by western blot (A). The fourth sequence was used for further assays in which shRNA, 3BP2 shRNA and NT (non-target) shRNA-transduced HMC-1 cells were starved overnight without FBS. On the following day, cells were stimulated with 100 ng/ml SCF for 5 and 15 min. Cells were lysed as described elsewhere (18). Western blot was performed with the indicated antibodies (B). Quantification of band intensity for each blot was performed by densitometry. The data are representative of three independent assays. FACS analysis of KIT expression was carried out in NT and 3BP2shRNA HMC-1 cells (C). Percentage expression and mean fluorescence, in parentheses, are indicated in the figure.

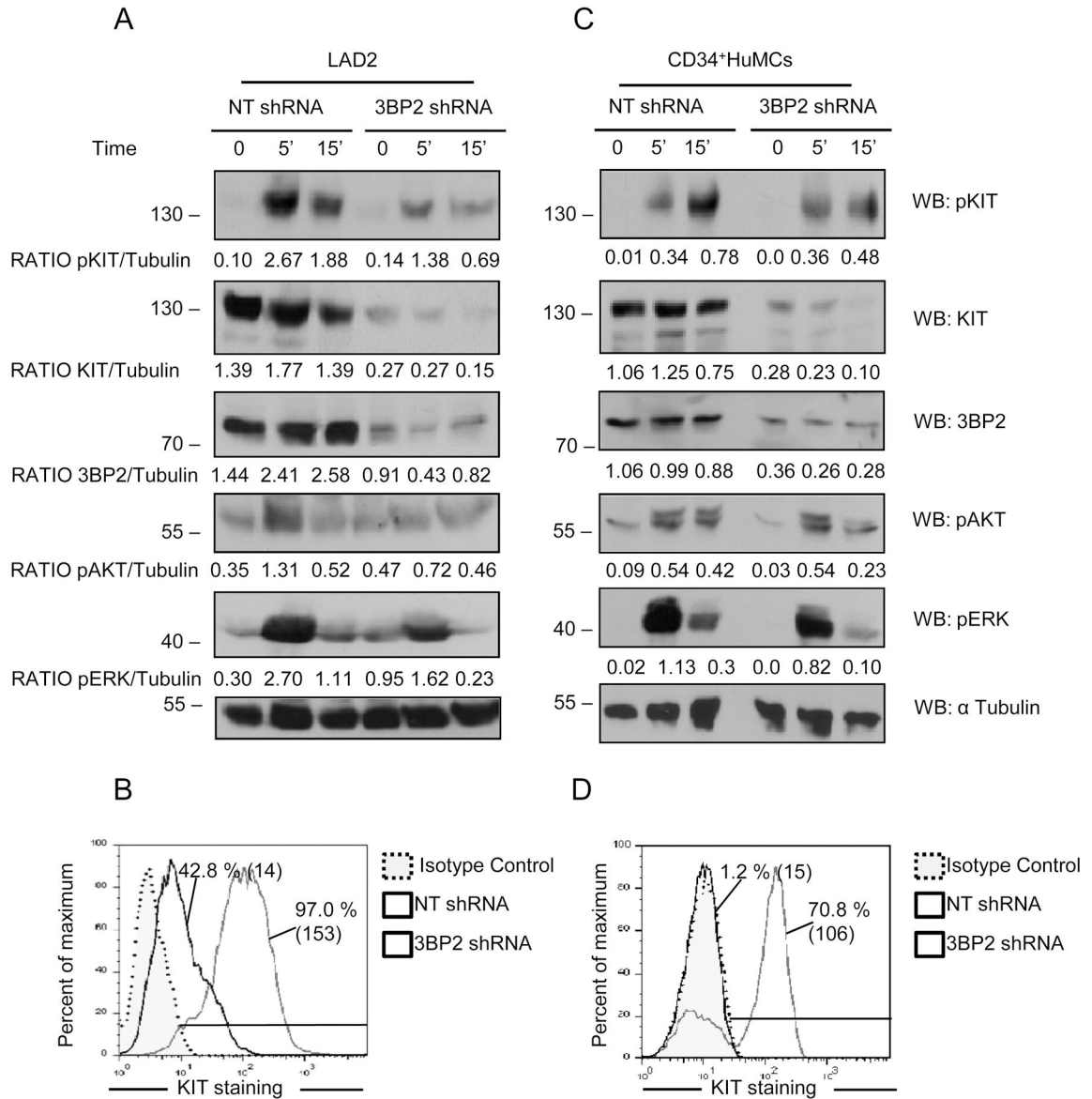


Figure 2. 3BP2 silencing reduces KIT expression and signals in human mast cells
 LAD2 cells (A) and CD34⁺huMCs (C) were transduced with either control NT (non-target) shRNA or 3BP2 shRNA and then starved in culture media without SCF. The following day, cells were stimulated with 100 ng/ml SCF for the indicated times. Western blots probed with the following antibodies: anti-phospho KIT, anti-KIT, anti-3BP2, anti-phospho AKT, anti-phospho ERK and anti- α tubulin (used as loading control) (A). Band intensity for each blot was quantified by densitometry. The data are representative of three independent assays. FACS analysis of KIT expression was carried out in NT and 3BP2shRNA in LAD2 cells (B) and CD34⁺ huMCs (D). Percentage expression and mean fluorescence, in parentheses, are indicated in the figure.

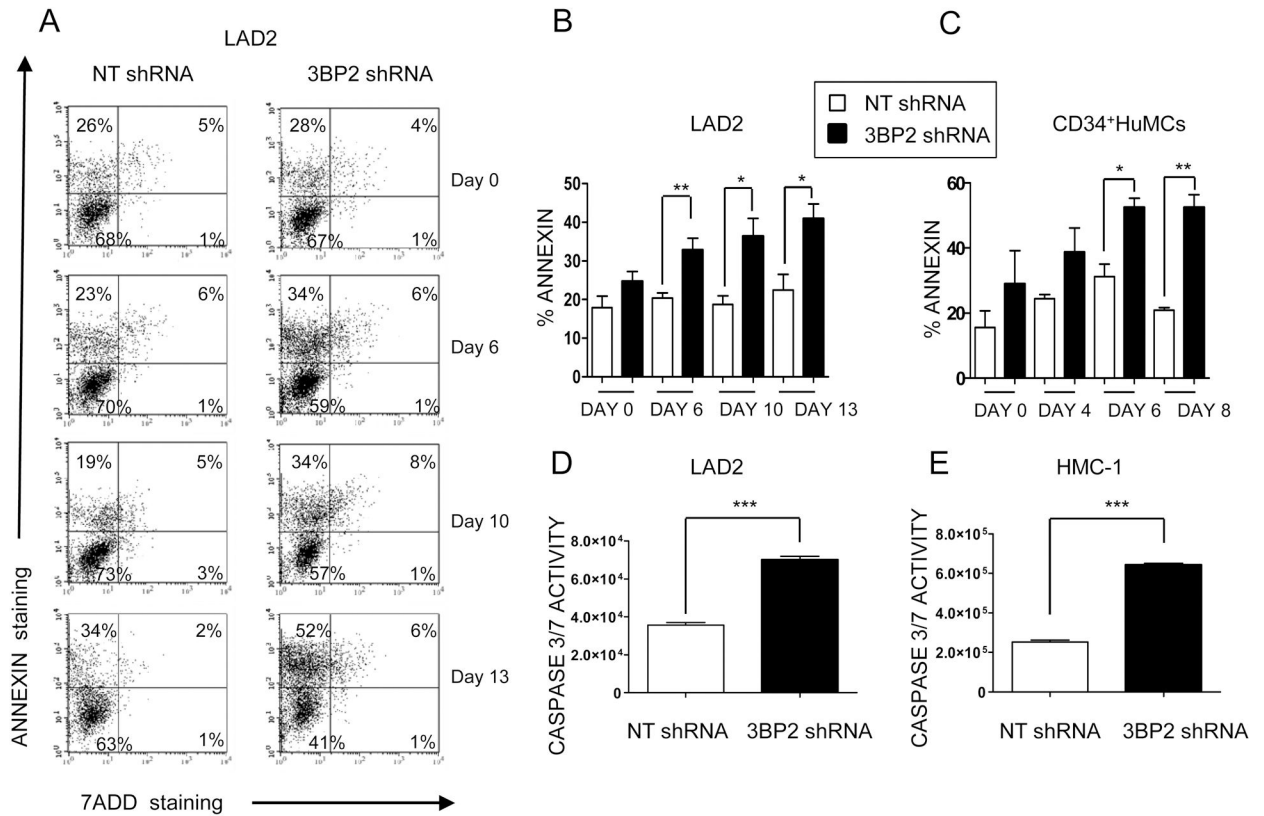


Figure 3. 3BP2 silencing increases mast cell apoptosis and caspase 3/7 activity

Dot blot of 7ADD and Annexin V staining of NT (non-target) and 3BP2-silenced LAD2.

Apoptotic cells were gated from the Annexin V⁺ 7AAD⁻ cells (A). Graphic bars represent the percentage of apoptotic cells measured by Annexin V staining. The results are the mean of three independent experiments using NT (non-target) shRNA and 3BP2 shRNA transduced LAD2 cells (B) and CD34⁺ huMCs (C). Statistical significance (* $p < 0.05$, ** $p < 0.01$) is relative to NT shRNA. Caspase 3/7 activity was performed at day 6. 3BP2 silencing increased caspase 3/7 activity in LAD2 (D) and HMC-1 cells (E). The data show the mean of three independent experiments. Statistical significance (***) $p < 0.001$ is relative to NT shRNA.

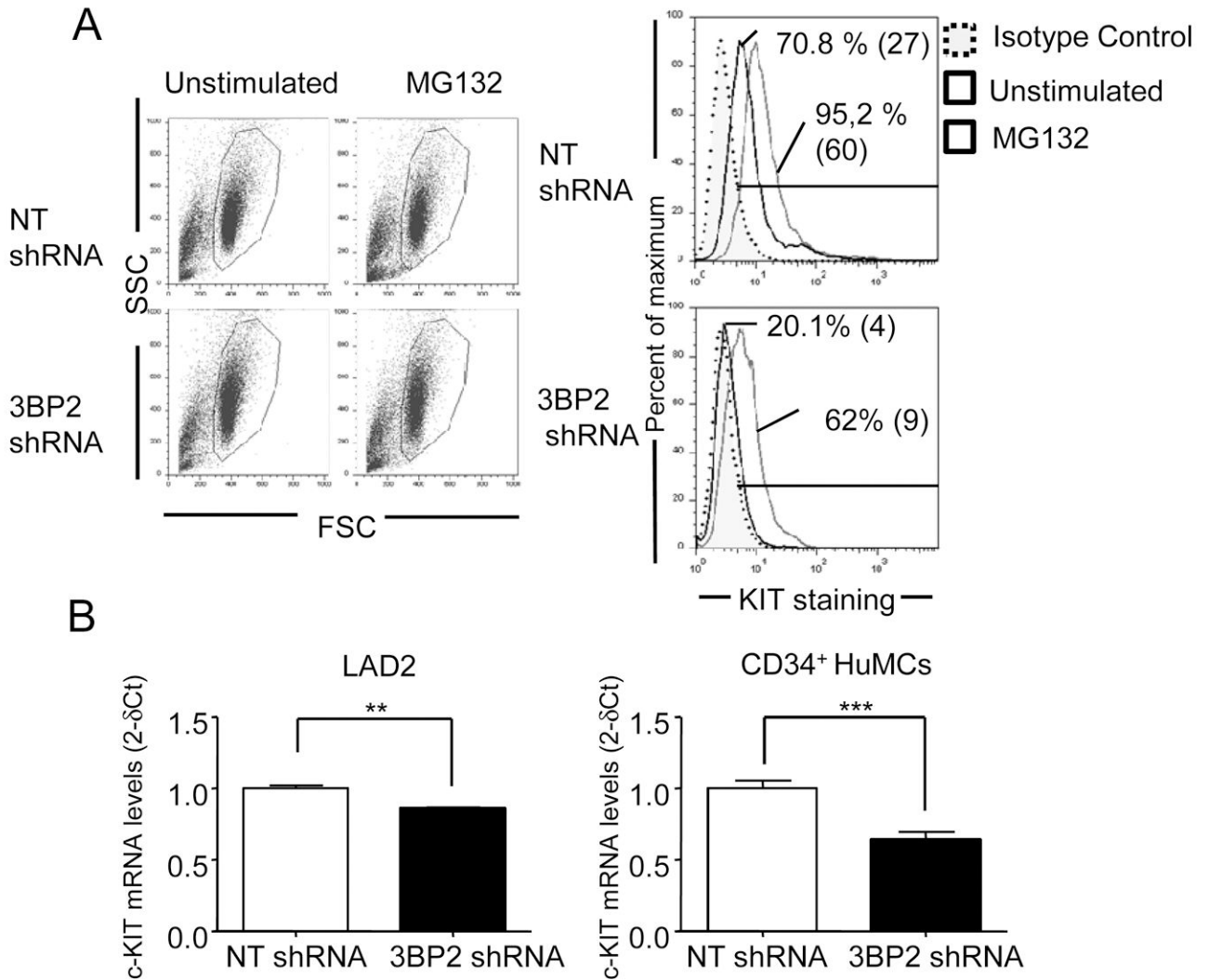


Figure 4. Silencing of 3BP2 results in decreased KIT mRNA

NT (non-target) shRNA and 3BP2 shRNA transduced LAD2 cells were treated with the proteasome inhibitor MG132 or vehicle (DMSO) for 3 hours. Forward Scatter (FSC) and Side Scatter (SSC) dot plots from all cases are shown (left panel). Cells were then stained for KIT and gated cells were analyzed by flow cytometry (right panel) (A). Percentage expression and mean fluorescence, in parentheses, are indicated in the figure. Real-time PCR was performed in LAD2 (B) and CD34⁺-derived huMCs (C) from 3BP2 and NT shRNA transduced cells using c-kit as a probe. The data are the mean of three independent experiments and in the case of CD34⁺ HuMCs, from three different donors. Significant differences (***) $p < 0.001$ were found between NT (non-target) and 3BP2 shRNA transduced shRNA huMCs.

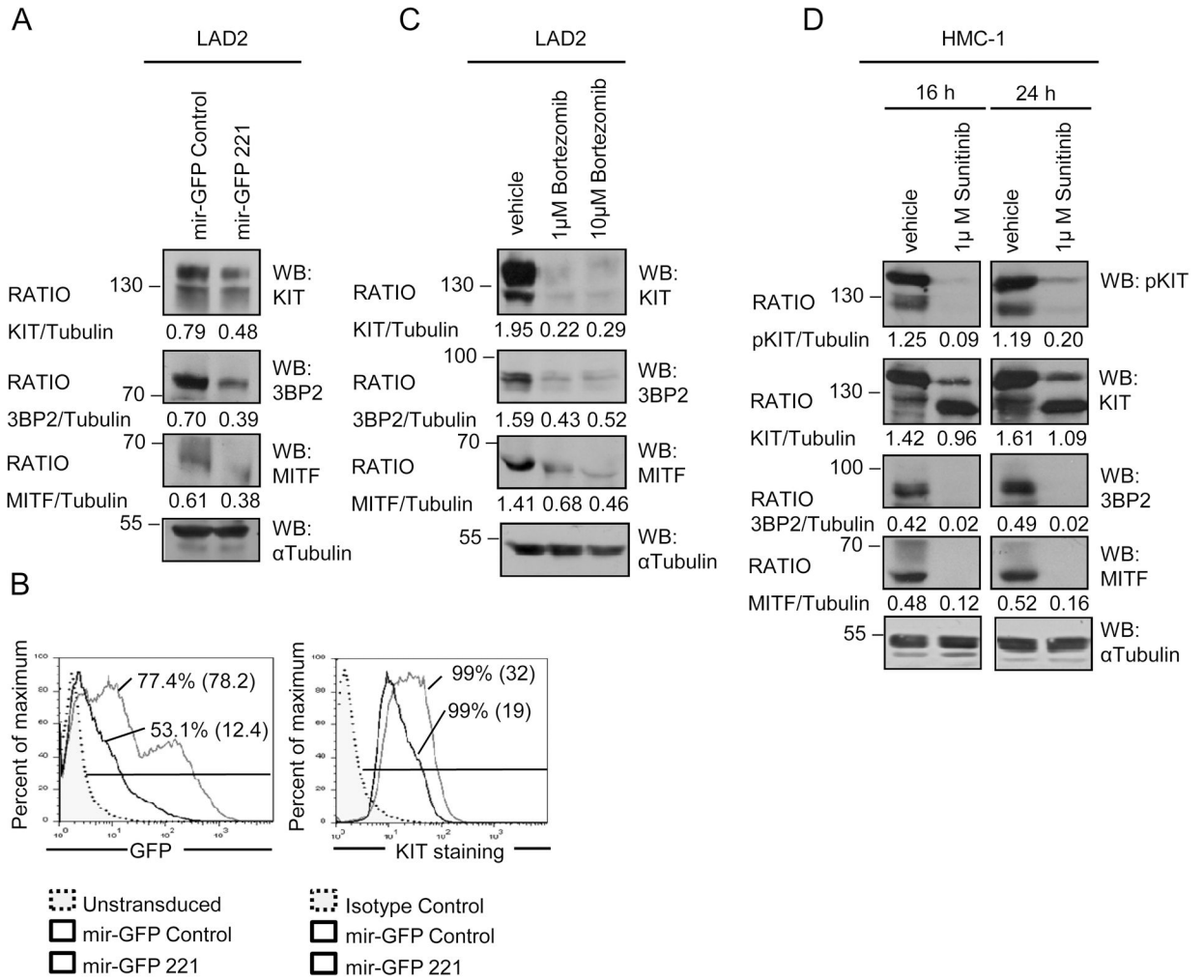


Figure 5. Reduced KIT expression and kinase inhibition lead to reduced 3BP2 and MITF expression in LAD2 and HMC-1 respectively

Mir-GFP Control and mir-GFP 221 transduced LAD2 cells were selected with puromycin, and after 2 weeks, western blots were performed with specific antibodies as indicated in the figure (A). Band intensity was quantified by densitometry. Levels of infection, measured by GFP expression were analyzed by FACS (left panel), GFP⁺ cells were gated and analyzed for KIT expression (right panel) (B). Percentage expression and mean fluorescence, in parentheses, are indicated in the figure. LAD2 cells were treated with the proteasome inhibitor, bortezomib, for 24 hours. Western blots were performed with specific antibodies as indicated in the figure (C). Inhibition of KIT tyrosine kinase activity with sunitinib was carried out in HMC-1 cells, in which KIT is constitutively activated. KIT phosphorylation, KIT, 3BP2 and MITF expression were analyzed by western blot with specific antibodies. αTubulin was used as the loading control (D). Densitometric quantification of band intensity is shown. The data are representative of three independent experiments.

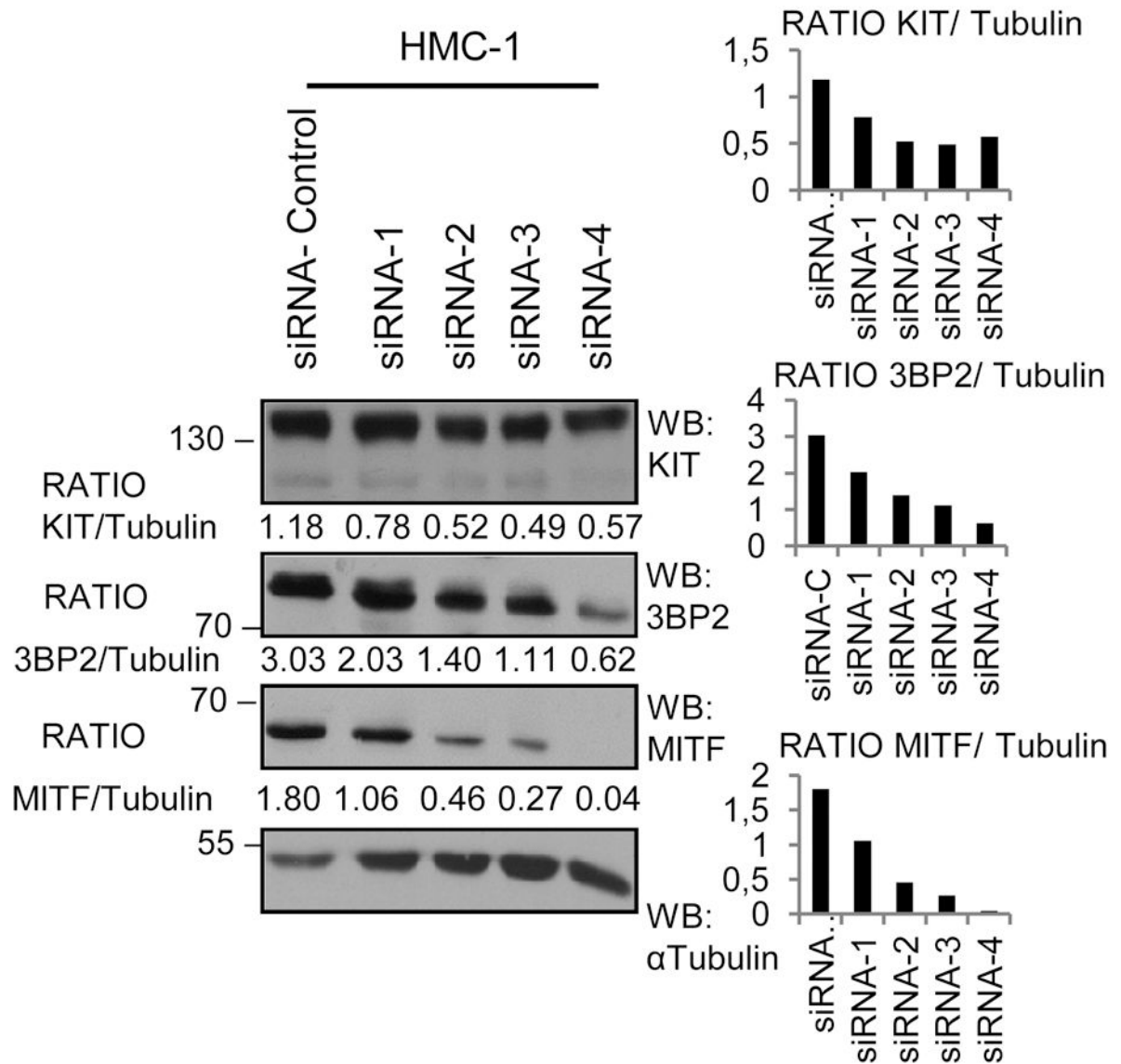


Figure 6. MITF expression decreases after 3BP2 silencing

MITF expression was evaluated after 3BP2 silencing in HMC-1 using various siRNA sequences as described in material and Methods section. Western blots of whole cell lysates were performed with anti-KIT, anti-3BP2, anti-MITF and anti- α tubulin. Band intensity was quantified by densitometry and is represented by bars in the righthand panel.

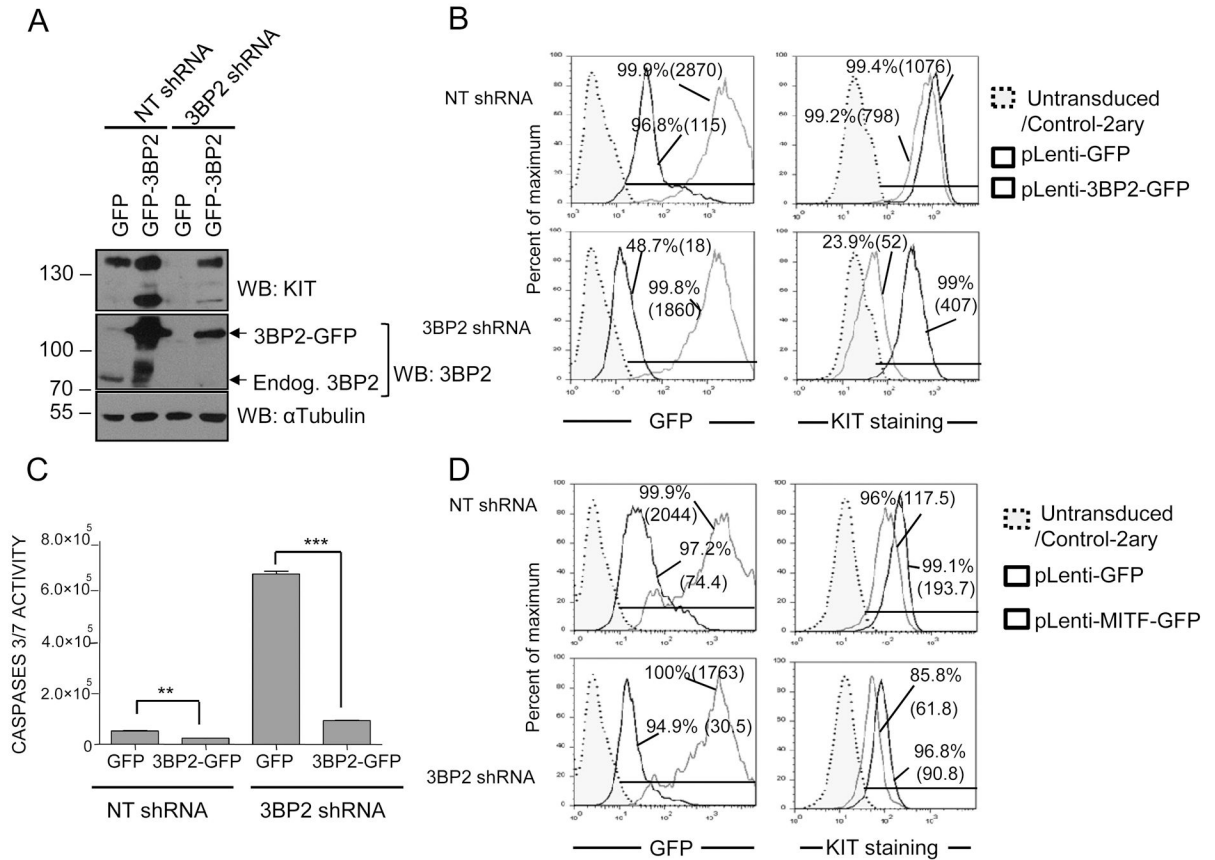


Figure 7. 3BP2 reconstitution restores KIT expression and cell survival. MITF reconstitution upregulates KIT in 3BP2 silenced cells

NT (non-target) shRNA and 3BP2 shRNA transduced LAD2 cells were infected with the chimera 3BP2-GFP or GFP lentiviral particles (as control). 3BP2-reconstituted levels and KIT expression were analyzed by western blot with specific antibodies in day 6 after 3BP2-reconstitution (A). GFP transduction levels and KIT surface expression were assayed by flow cytometry (B). Caspase 3/7 activity was determined in day 6 after 3BP2-reconstitution (C). NT (non-target) shRNA and 3BP2 shRNA transduced LAD2 cells were infected with the chimera MITF-GFP or GFP lentiviral particles (as control). MITF-reconstituted levels and KIT expression were analyzed by western blot with specific antibodies in day 6 after MITF-reconstitution (D). The data show the mean of three independent experiments. Significant differences (** $p < 0.01$, *** $p < 0.001$) were found between GFP or 3BP2-GFP versus NT (non-target) and 3BP2 shRNA reconstituted LAD2 cells. Percentage expression and mean fluorescence, in parentheses, are indicated in the FACS histograms.